A 01

CODING FORMS FOR SRC INDEXING

Microfiche No.	
OTS0573868	
New Doc ID	Old Doc ID
86990000018	
Date Produced	Date Received	TSCA Section
10/10/98	12/09/98	8D
Submitting Organization	
INTL ISOCYANATE INSTIT INC	
Contractor	
CARNEGIE MELLON UNIVERSITY	
Document Title	
INVESTIGATION OF THE CHEMISTRY OF TDI IN BIOLOGICAL MATERIALS, WITH COVER LETTER DATED 12/03/1998	
Chemical Category	
BENZENE 2,4 DIISOCYANATE-1-METHYL 2,4-TOLUENE DI* (584-84-9)	

A 02

OFFICE OF TOXIC SUBSTANCES

REV. 7/27/82

CODING FORM FOR GLOBAL INDEXING

Microfiche No. (7) •		1	No. of Pages		2		
Doc I.D.		3	Old Doc I.D.	8699000018	4		
Case No.(s)					5		
Date Produced (6)	6	Date Rec'd (6)	7	Conf. Code •	8		
				N			
Check One: ☐ Publication	☐ Internally Generated	☐ Externally Generated					
Pub/Journal Name					9		
					9		
Author(s)					10		
Organ. Name					11		
Dept/Div					12		
P.O. Box	13	Street No./Name			14		
City	15	State	16	Zip	17	Country	18
MID No. (7)	19	D & B NO. (11)			20		
Contractor					21		
Doc Type					22		
• ___ • ___ • _____	8D	___	___	___			
Doc Title					23		
Chemical Name (300 per name)			25	CAS No. (10)	24		

A 03

13217

INTERNATIONAL ISOCYANATE INSTITUTE, INC.
201 Main Street, Suite 403 • La Crosse, WI 54601 • 608/796-0880 • FAX 608/796-0882

December 3, 1998

TSCA Document Processing Center (TS-790)
Office of Pollution & Toxics
Environmental Protection Agency
401 M Street, SW
201 East Tower
Washington, DC 20460

Contains No CBI

RECEIVED
OPPT NCIC
98 DEC -9 PM 3:09

Attn: 8(d) HEALTH 7 SAFETY STUDY REPORTING RULE
(REPORTING)

Dear Sir or Madam:

We herewith submit a copy of the following recently completed health and safety study: *"Investigation of the Chemistry of TDI in Biological Materials."*

RECEIVED
OPPT NCIC
99 JAN 11 AM 9:09

Name of Chemical Substance:	benzene 2,4 dissocyanate-1-methyl
Common name:	2,4-toluene diisocyanate
Chemical Abstracts Service Number:	584-84-9
Abbreviation:	2,4-TDI
Name of Chemical Substance:	benzene, 1,3-diisocyanatomethyl-
Common name:	generic toluene diisocyanate
Chemical Abstracts Service Number:	26471-62-5
Abbreviation:	2,4-TDI and 2,6-TDI (mixture)
Authors	A.L. Kennedy and W.E. Brown

The International Isocyanate Institute (III) project identification number (11320) has been marked on the title page of the report. Please refer to the III identification number in any communication regarding this study. The enclosed report does not contain any Confidential Business Information.

This study was sponsored by the International Isocyanate Institute on behalf of the following:

The Dow Chemical Company
Bayer Corporation
BASF Corporation
ICI Americas, Inc.
Lyondell Chemical Company

86990000018

Very truly yours,

M.J. Blankenship
Managing Director

Investigation of the Chemistry of TDI in Biological Materials

Final Report

Project ID: AM-AB-80

11320

10 October 1998

A.L. Kennedy and W.E. Brown

Department of Biological Sciences

Carnegie Mellon University

4400 Fofth Avenue

Pittsburgh, Pennsylvania, USA

PH: (412) 268-3416

FAX: (412) 268-1811

Sponsor

International Isocyanate Institute, Inc

Table of Contents

List of Figures and Tables

Figures

Tables

SUMMARY

The objective of this study was to investigate the fate of toluene diisocyanate (TDI) in biological matrices due to its chemical reactivity. A number of variables were tested for their effect on the reaction of TDI with biological materials. Route of administration (vapor vs liquid), pH, composition of biological materials (gastric simulans, lung lavage, plasma and pure proteins) were test variables. Modification of serum albumin by TDI correlates linearly with TDI concentration when added either as a liquid or a vapor. The very efficient reaction between TDI and protein over a wide range (1:1 to 48:1) of TDI:Protein ratios at pH 7.4 indicates that the reaction with protein competes successfully with the hydrolysis of TDI to the diamine. In the vapor phase the uptake of the TDI into the aqueous phase is independent of the biological material in the aqueous phase. Incorporation of TDI into protein at pH 7.4 following vapor exposure is directly related to the concentration of TDI in the aqueous phase indicating a very efficient reaction. Lung lavage shows less specificity with toluene diamine being produced during the reaction. While TDI appears in the aqueous phase during vapor exposure at pH 2.3, there is no incorporation of the TDI into protein but rather hydrolysis to toluene diamine. Using affinity chromatography, it was observed that there was a direct correlation between the degree of modification and the extent to which the albumin component is recognized by the affinity ligand used in the chromatography. This would indicate that TDI can be classified as an affinity label for the high affinity ligand site on albumin thus accounting for the successful labeling in competition with the hydrolysis. The appearance of diamine in the reaction mixture after modification of albumin at high isocyanate:protein ratios supports a saturation reaction endpoint. Several observations were possible from the SDS PAGE molecular weight analysis of the reaction mixtures. Increased modification caused a shift in electrophoretic mobility to lower molecular weight following by appearance of high molecular weight species (dimers, trimers etc.). Reaction at lower pH did not alter the structure of molecules nor result in incorporation of TDI into macromolecules. There is protein modification of all biological samples at pH 7.4 and none at pH 2.3. Vapor modification of proteins showed significantly different structural effects on proteins to that seen by liquid modification.

INTRODUCTION

The objective of this study was to investigate the fate of TDI in biological matrices due to its chemical reactivity.

The chemistry and biochemistry of isocyanate compounds have been reviewed (Brown, 1986; Brown and Kennedy, 1989). However, as yet, a systematic investigation is not available on the quantitative, qualitative and kinetic aspects of the reactivity of these compounds in biological matrices such as aqueous buffer solutions, protein model systems, blood, plasma, urine and stomach contents. A limited study was performed to analyze the in vitro reactivity of the 2,6 isomer of TDI in fluids such as stomach contents and sera (Jeffcoat, 1985). In both fluids, the half-life of the 2,6 TDI was 2 minutes or less and the reactivity was dependent on the delivering solvent. This data has been extended through a systematic investigation of each of the individual isomers of TDI and of the commercial 80/20 2,4/2,6-TDI isomer mixture. It is also important to address not only the loss of reactive isocyanate but also the fate of the reactive compound. Detailed knowledge of the specificity and kinetics of the reactivity of these compounds under aqueous conditions will help to define the behavior of TDI in animal and man.

Toluene diisocyanate (TDI) is a highly reactive compound that has been widely used in the production of polyurethane foams

and coatings. Numerous studies have been performed with TDI both *in vitro* and *in vivo* to evaluate the effects associated with exposure. Due to the limited solubility of TDI under aqueous, neutral pH conditions, many *in vitro* studies have included the use of dimethylsulfoxide (DMSO) (Mapp et al., 1991) or acetone (Karol *et al.*, 1978) to provide a miscible delivery solvent. Several *in vivo* studies have also required the use of delivering agents such as corn oil (Jeffcoat, 1985, Timchalk *et al.*, 1993) or di-n-butyl ether (Rosenberg and Savolainen, 1985). In addition to solvent differences, several routes of animal model exposure have also been examined for TDI which include: oral (Jeffcoat, 1985; Timchalk *et al.*, 1993), percutaneous (Rosenberg and Savolainen, 1985), and inhalation routes (Kennedy *et al.*, 1989). One of the major questions remains as to what effect if any, route of administration and solvent variations have on the experimental results obtained when the reactivity of the compound is involved. Of note is the catalytic effect DMSO has on the hydrolysis of isocyanates in water (Gahlmann et al, 1993). A striking difference in experimental endpoints was observed, for example, in the carcinogenicity testing for TDI. Following oral administration, tumor development was observed (Dieter *et al.*, 1990); however, a similar effect was not seen in the inhalation study (Loeser, 1983). This is just one instance where the chemical endpoint of TDI may be influenced by either the route of delivery or the solvent in which it is delivered and thus contributes to the experimental outcome.

Isocyanates have the potential to react with amino, carboxyl, sulfhydryl, imidazole and hydroxyl functional groups, all of which are present on a variety of biological macromolecules. A detailed review of this chemistry has been given (Brown *et al.*, 1987). One of the principle reactions of interest, in terms of health and environmental concerns, is the reaction of the isocyanate with water. When TDI enters an aqueous environment such as the respiratory tract or buffered protein solution, it has the potential to react with the water to yield toluenediamine (TDA) and carbon dioxide. TDA, itself is a known carcinogen in rodents (NCI, 1979) when administered by gavage in corn oil. As presented above, carcinogenicity testing with TDI itself, is not definitive and shows that tumor development was related to route of exposure. Timchalk and co-workers (1993) have shown differences in the amount of TDA and its metabolic products in urine following TDI exposure by both oral or inhalation routes.

Competing with the hydrolysis and self-polymerization reactions, the isocyanate may alternatively react with macromolecules present in the various environments. Biochemical studies of tissues and fluids from *in vivo* exposures to TDI have shown that conjugation reactions predominated, but that the competing reactions were altered by route of animal exposure (Kennedy, *et al.*, 1993A). Jeffcoat (1985) performed a limited study of the *in vitro* reactivity of 14C-2,6-TDI in biological materials which included serum and stomach contents. In both fluids, the rate of TDI loss was measured and found to be complete within 2 min. under the experimental conditions studied. Analysis of reaction products was not included.

The present study was undertaken to characterize further the reactivity of TDI *in vitro* by analyzing not only reaction products but also the effect of method of administration on the endpoints of the reactions (Table I). This work provided data on the question of TDA formation during and following TDI exposure. The overall goal was to use this data to aid in the understanding of the underlying reactions and rates which may effect the endpoints observed in *in vitro* and *in vivo* studies.

Table I. *In Vitro* Matrix of Experiments

Title		Liquid Addition	Vapor Exposure
Test Solutions	pH		
Blood	7.4	+	1.8 ppm
Plasma	7.4	+	0.2, 1.8 ppm
Lavage	7.4	+	0.2, 1.8 ppm
Gastric Simulans	2.3	-	0.2, 0.5 ppm
Rat Serum Albumin	7.4	1, 10,50 :1 molar ratio	0.2, 0.5, 2.4 ppm
Guinea Pig Serum Albumin	7.4	1, 10, 25,	-

		100:1 molar ratio	
Test Conditions			
Variable concentration		1, 10, 25, 50, 100:1 molar ratio	0.2, 0.5, 1.8 ppm
TDI in Acetone		+	-
Reaction Time			
30 Minutes		+	-
1 Hour		-	+
24 Hours		+	-
Biochemical Analysis			
Molecular Sieve	Centricon (1)	+	+
Fractionation	BioGel-P (2)	+	+
RP-HPLC/TLC (3)		+	+
Affinity Chromatography		+	+
SDS PAGE (4)		+	+

1. Filtration separation unit by Amicon

2. Gel filtration matrix material by BioRad

3. Reverse Phase-HPLC/Thin Layer Chromatography

4. SDS polyacrylamide gel electrophoresis

MATERIALS AND TEST DESIGN

Chemicals. The 2,4-TDI isomer and the 80/20 mixture of 2,4- and 2,6-TDI was used with 14C in the benzene ring. The radiolabeled TDI was supplied by Sigma Chemical Company and New England Nuclear. Determination of the specific activity of each sample is given below.

Quantitation of Reactive Isocyanate Concentration. To determine the concentration of reactive TDI in the stock solutions of TDI, an aliquot of each TDI stock solution was spotted directly on a PNBPA impregnated glass fiber filter. The resulting PNBPA derivative was extracted from the filter with a one-milliliter aliquot of HPLC grade acetonitrile and analyzed by HPLC according to the procedure of Schroeder and Moore (1985). HPLC analysis of the PNBPA derivative yielded an average recovery of radioactivity from the HPLC column of 100.1 ± 2.8% (n=7) and a recovery of the combined 2,4/2,6 isomers of TDI at 95.4 ± 1.5% (n=11). For the Sigma 14C-TDI, the distribution of isomers as determined by collection and liquid scintillation analysis of the respective isomers following their elution from the HPLC column yielded 85.0 ± 4.0% (n=26) 2,4- isomer while the New England Nuclear (NEN) 14C-TDI was found to be 100% 2,4- isomer.

Determination of 14C-TDI specific activity. To determine the specific activity of the reactive isocyanate in each TDI sample used, liquid scintillation analysis of the HPLC purified PNBPA derivatives of TDI was performed. For the Sigma

14C-TDI sample the specific activity was determined to be 23.7 ± 0.4 (n=6) mCi/mmole when data from the direct analysis of neat TDI and data from the vapor generated TDI were combined. For the NEN 14C-TDI sample the specific activity was determined by the manufacturer to be 12.5 mCi/mmole.

Preparation of Blood and Plasma. Two, Fisher 344 rats were anesthetized with Beuthanasia and whole blood was collected via cardiac puncture into vacutainers containing sodium citrate anti-coagulant. The blood sample was divided and 60% was separated into plasma and cell components. The samples of whole blood and plasma were stored at 4°C until used in the vapor and liquid titration experiments.

Preparation of Lung Lavage. Tracheostomy was performed on the lungs of the two control rats and each lung was lavaged with 5 ml of phosphate buffered saline (PBS), pH 7.4. The lavage fluid from the two animals was combined and stored at 4°C.

Preparation of Gastric Simulans. Dr. H.-D. Hoffmann (BASF, Ludwigshafen, Germany) provided a formulation for Gastric Simulans. Its composition was 2.0 gm NaCl, 3.2 gm pepsin, 7 mL 12 N hydrochloric acid to a final volume of 1 liter with water. The measured pH of the final solution was 1.8.

Rat and Guinea Pig Serum Albumin. Rat serum albumin (RSA) and guinea pig serum albumin (GPSA) were purchased from Sigma (grade Fraction V) and used without further purification.

In Vitro Titration - Liquid Phase Exposure. All liquid-phase reactions were allowed to react for 30 minutes under the experimental conditions given below.

Experiment #1: Liquid phase reactions were performed in 24 well tissue culture plates. 1 mL of each stock solution was placed in the appropriate well. After addition of a constant volume (20 µL) of either pure acetone or TDI acetone stock solution to each well, the samples were reacted for 30 minutes with agitation at room temperature. At the conclusion of the reaction, each sample was removed and transferred to a 1.5 mL microfuge tube. Immediately, 25 µL was aliquoted to a scintillation vial containing 5 mL of CytoscintTM (liquid scintillation counting cocktail), 25 µL was aliquoted to a glass scintillation vial and digested with NCSTM tissue solubilizer (Amersham), 50 µL was applied directly to a PNBPA impregnated filter to derivatize any remaining free TDI, 50 µL was added to a solution of 0.01 N HCl to lower the pH to 2, and the remainder was frozen.

Experiment #2: Liquid phase reactions with RSA and GPSA were repeated at a concentration of 1 mg/mL protein with variable ratios of isocyanate to protein. Proteins were dissolved in PBS, pH 7.4, and 1 mL aliquots were distributed in 20 mL, screwcap glass vials. Three different ratios of isocyanate to protein were tested and each experiment was performed in duplicate. At time zero, an aliquot of stock TDI in acetone was added and the sample was stirred for 30 minutes. At the termination of the experiment, all samples were treated as described in Experiment #1.

Modification at Variable Molar Ratio of TDI:Protein.

Experiment #1: Assuming an average protein concentration of 100 mg/mL for blood and plasma, 10 mg/mL for lavage fluid and a measured concentration of 1 mg/mL for pure proteins, the initial experiments were performed at a molar ratio of 1 mole of TDI added per mole of protein. To accomplish this, stock solutions were generated by first diluting 2.85 µL of neat 14C-TDI in 200 µL of dry acetone (Stock A, 99 mM TDI). Subsequently, 20µL of Stock A was added to 180 µL dry acetone (Stock B, 9.9 mM TDI) and finally 10 µL of Stock B was added to 90 µL of dry acetone (Stock C, 0.99 mM TDI). 20 µL of Stock A was added to 1 mL of blood and plasma, 20 µL of Stock B was added to 1 mL of lavage fluid and 20 µL of Stock C was added to 1 mL of RSA solution to achieve a 1:1 molar ratio in each case. A control for each test solution involved addition of 20 µL of dry acetone to separate 1 mL aliquots of the respective test solutions.

Experiment #2: In this experiment, all serum albumin concentrations were 1 mg/mL. Separate stock solutions of TDI were generated just prior to reaction. The concentration of the stock solutions was calculated such that no more than 40 µL of acetone would be present in any reaction mixture. To determine the specific activity of each stock solution, 10 µL of each was taken for scintillation analysis, and 20µL was spotted directly on a PNBPA filter. Stock solutions were made as follows: 4 µL neat 14C-TDI was added to 800 µL HPLC grade, dry acetone (Stock D, 35mM TDI), 100µL Stock D was added to 900 µL dry acetone (Stock E, 3.5 mM TDI), and 100 µL Stock E was added to 900 µL dry acetone (Stock F, 0.35 mM TDI).

Analysis of acetone stocks for reactive TDI. Each set of stock isocyanate solutions was made freshly for each experiment. Immediately upon generation, an aliquot of each stock solution was added directly to a PNBPA saturated

glass fiber filter, reacted for 45 minutes, extracted with acetonitrile and analyzed by HPLC.

Experiment #1: HPLC analysis of the stock solutions A, B, and C showed that the distribution of isomers for these samples was 85.2% 2,4-TDI and 14.8% 2,6-TDI. Combining the liquid scintillation data and the quantitation of µg TDI present using an area integration from a standard curve, the specific activity was confirmed to be 23.7 mCi/mmole.

Experiment #2: HPLC analysis of stock solutions D, and E showed that the TDI was 95.2 ± 2.6% pure as assessed by recovery of material associated with the 2,4 and 2,6 isomers of TDI. The isomer distribution was found to be 84.1 ± 0.3 % 2,4-TDI by integration of the separated PNBPA derivatives on HPLC.

In Vitro Titration - Vapor Phase Exposure Protocol. One milliliter of each test sample for vapor exposure was aliquoted in quadruplicate into 24 well tissue culture plates (Falcon). The fluid filled plate was placed into a pre-equilibrated glass plethysmograph exposure chamber. All vapor exposures were performed in a dynamic system as diagrammed in Figure 1. The atmosphere was pulled across the plate at a flow rate of 10 liters/minute. The vapor was introduced into the central chamber via an impinger that was regulated by an inline flowmeter at rates ranging from 0.4 to 5 liters/minute, depending on the target concentration. Following one-hour exposure in every experiment, the chamber was exhausted at a rate of 50 liters/minute for 5 minutes. The plate was removed and the samples were transferred to 1.5 mL microfuge tubes and stored at -60°C. Prior to freezing, samples were removed for analysis.

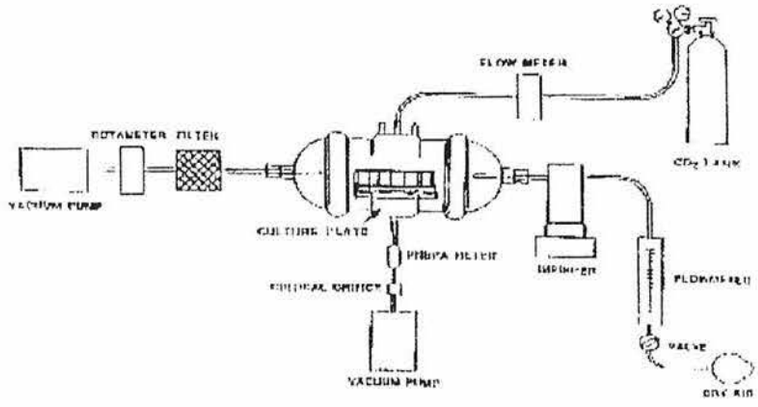

Figure 1. Schematic of vapor exposure apparatus.

RESULTS.

Atmospheric Monitoring During Vapor Exposure. Throughout the exposure, atmospheric samples were collected directly from the chamber either onto PNBPA-coated glass fiber filters or into an impinger, containing Marcali trapping solution (Marcali, 1957), at an airflow rate of 2 liters/minute. Quantitation of atmospheric TDI concentration was performed by Marcali assay (Marcali, 1957) as modified by NIOSH (1978) and by HPLC analysis of the PNBPA filter extract as previously described (Kennedy *et al*, 1989). Both assay fluids were counted and the radioactivity was used as an additional means to measure the concentration. Table II summarizes the final concentrations for each of the vapor exposure experiments performed.

Table II. Determination of 14C-TDI Concentration during Vapor Exposure

Method	n	Concentration (ppm) (mean±SD) Average	Concentration (ppm) (mean±SD)

Exp #1 (2,4 TDI)			
PNBPA Specific Activity	2	0.36	
PNBPA HPLC Assay	2	0.23	0.21 ± 0.13
Marcali Specific Activity	2	0.11	
Marcali Assay	2	0.08	
Exp #2 (2,4/2,6 TDI)			
PNBPA Specific Activity	8	1.69 ± 0.55	
PNBPA HPLC Assay	8	1.80 ± 0.79	1.79 ± 0.19
Marcali Specific Activity	4	1.60 ± 0.36	
Marcali Assay	4	2.05 ± 0.39	
Exp #3 (2,4 TDI)			
PNBPA Specific Activity	2	0.68	
PNBPA HPLC Assay	2	0.52	0.55 ± 0.1
Marcali Specific Activity	2	0.55	
Marcali Assay	2	0.44	

Characterization of Reaction Products:

Post-Reaction Derivatization of PNBPA. Aliquots of each vapor and liquid modification were spotted directly onto PNBPA saturated glass fiber filters. Extracts of the filters were tested by HPLC. In all cases the results were negative for the presence of any reactive free TDI as determined by the absence of a PNBPA derivative following the reaction protocols.

Post-Reaction Quench with HCl. Aliquots of each vapor and liquid modification were mixed with equal volumes of concentrated hydrochloric acid to lower the pH and thus prevent the reversal of any sulfhydryl group modification that may have occured. These samples were saved for future analysis if deemed necessary.

Post-reaction Analysis for Radioactivity. After each reaction an aliquot of the reaction mixture was added directly to a scintillation vial. Five milliliters of counting cocktail (Cytoscint ES) was added, the samples mixed and subjected to liquid scintillation analysis on a Beckman Scintillation Counter.

Results of determination of total radioactivity in each sample are given in Tables III and IV. The cpm's measured for each sample were converted to nanomoles/mL using the measured specific activity for the isocyanates used in each case and a counting efficiency of 85% measured for the specific scintillation counter used.

Table III. Analysis of Liquid Reaction Mixtures

Sample Name	Sample	Protein Conc. (mg/mL)	Molar Ratio TDI/Protein	n	Mean nanomoles/mL
LB2	Blood	100	1:1	1	1985
LP2	Plasma	100	1:1	2	2753
LR14	RSA	1	1:1	4	17.6 ± 0.5
LR16	RSA	1	10:1	4	156.1 ± 11.7
LR18	RSA	1	50:1	4	710.8 ± 76.7
LR21	RSA	1	25:1	2	435.5
LG1	GPSA	1	1:1	2	14.10
LG2	GPSA	1	25:1	2	449.0
LG4	GPSA	1	1:1	2	18.10
LG5	GPSA	1	10:1	2	164.5
LG6	GPSA	1	100:1	2	796.5
LL	Lavage	10	1:1	4	37.91 ± 3.8

1. Assumed concentration with average protein molecular weight = 50 kDa.

2. Assumed concentration with average protein molecular weight = 50 kDa.

Table IV. Analysis of Vapor Reaction Mixtures

Sample Code	Sample	Protein Conc. (mg/mL)	TDI conc. (ppm)	pH	n	Mean nanomoles/mL
VB	Blood	100	1.79	7.4	2	4.20
VP	Plasma	100	1.79	7.4	8	36.3 ± 1.1
VR	RSA	1	1.79	7.4	8	37.2 ± 3.4
VL	Lavage	1	1.79	7.4	8	37.0 ± 3.6
VR	RSA	1	0.55	7.4	4	7.71 ± 0.9
VR	RSA	1	0.55	2.3	4	8.35 ± 0.04
VGS	Gastric Simulans	1	0.55	7.4	4	7.11 ± 0.08
VP	Plasma	1	0.21	7.4	4	3.78 ± 0.33
VL	Lavage	1	0.21	7.4	8	3.75 ± 0.31
VGS	Gastric Simulans	1	0.21	7.4	4	4.61 ± 0.25
VR	RSA	1	0.21	7.4	4	5.27 ± 0.17
VR	RSA	1	0.21	2.3	4	7.40 ± 0.80

Biochemical Analysis of End Products:

Centricon filtration. Centricon 10 (Amicon) is a membrane filter system for separation of high (>10 kDa) and low (<10 kDa) molecular weight molecules. They were used to separate the reaction products into conjugates of greater than 10kDa from the isocyanate hydrolysis product (diamines) and any other low molecular species resulting from the modifications. The results of each experiment are expressed as the percentage of the total radioactivity with a molecular weight greater than or less than 10 kDa. Table V is a compilation of molecular sieve separation data for the samples treated by the vapor exposure protocol.

Table V. Centricon fractionation of reaction products resulting from vapor exposure.

Sample Name	Sample	pH	% > 10 kDa
VP	plasma	7.4	99.9
VL	lavage	7.4	71.6
VR	RSA	2.3	8.1
VR	RSA	7.4	99.8

BioGel P2 column chromatography. BioGel P2 is a gel filtration column matrix that separates molecules on the basis of molecular weight. Using standard unlabeled serum albumin, it is possible to calibrate this column and thus determine the fraction of the radioactive label from the conjugation experiments that was associated with the protein fraction. The results are tabulated as the percentage of the total recovered radioactivity that was associated with the radioactivity. Sample chromatographic profiles are provided in Figure 2, a mixture of labeled serum albumin and KCl. These profiles represent the separation of the products in the reaction of guinea pig serum albumin (GPSA) with 14C-TDI. The modification is the result of adding an aliquot of a TDI/acetone stock solution to a 1 mg/mL solution of GPSA, pH 7.4, at a molar ratio of 25:1 (TDI:Protein). Integration of the peaks yields the fraction of material that is greater than 10 kDa under non-denaturing conditions (Figure 2, peak A) and the fraction that is less than 10 kDa (Figure 2, peak C). Table VI is a compilation of the results of the BioGel P2 analysis of samples from both the liquid and vapor phase exposure of biological fluids and proteins to TDI.

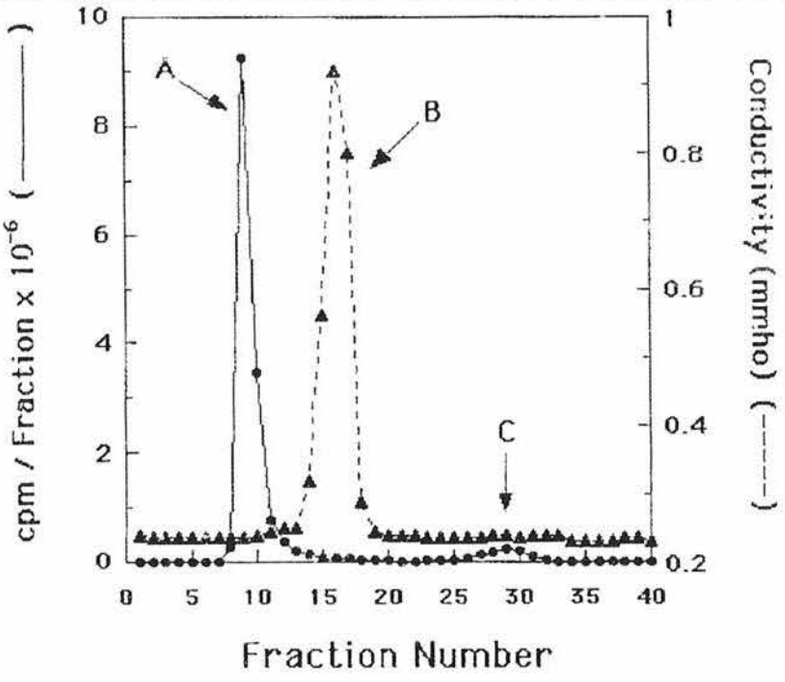

Figure 2. Representative profiles showing the molecular sieve separation of labeled macromolecules (A) from salt (B) and TDA (C) using BioGel P2 Chromatography. Integration of the peaks yields the fraction of labeled material that is greater than 10 kDa under native, non-denaturing conditions and the fraction of labeled material that is less than 10 kDa. These profiles represent the separation of the products of the reaction of GPSA with TDI. The TDI was added from an acetone stock solution at a molar ratio of 25:1 (TDI:Protein).

Table VI. BioGel P2 Separation of Reaction Products.

Sample	Exposure Condition	Molar Ratio TDI/Protein (1)	Sample Name	% of Recovered 14C in Peak A (Figure 2)
RSA	liquid	1.20	LR1	91
RSA	vapor	2.53	VR1	97
GPSA	liquid	0.96	LG1	96
GPSA	liquid	30.53	LG2	92
Plasma	liquid	1.38	LP2	95

1. Molar ratio of TDI to protein in the reaction mixture before separation.

Reverse-Phase HPLC and/or TLC. Thin layer chromatography (TLC) was performed on samples from the reactions to determine whether low molecular weight components were generated during the reactions of TDI with the biological macromolecules and fluids. Unlabeled TDA was used as a reference compound and identified by reaction with fluorescamine following development of the TLC plate. In all cases the developing solvent was butanol/acetic acid/water (20/80/20). Identification of the products resolved on the TLC plates was accomplished by fluorescamne (Sigma) derivatization and observation of the reacted plate under UV light and by autoradiography of the resulting TLC plate with Kodak X-Omat film. Table VII contains a list of the samples analyzed by TLC and the results of the presence or absence of a TDA co-migrating species in each of the reaction mixtures.

Table VII. TLC Analysis of Reaction Products.

Sample Name	pH	TDI conc. (ppm)	Sample	TDA
VR5	7.4	0.2	RSA	-
VR6	7.4	0.2	RSA	-
VR13	7.4	0.5	RSA	-
VR14	7.4	0.5	RSA	-
VR2	7.4	2.4	RSA	-
VR9	2.3	0.2	RSA	+
VR10	2.3	0.2	RSA	+
VR17	2.3	0.5	RSA	+
VR18	2.3	0.5	RSA	+
VL2	7.4	2.4	Lavage	+
VL5	7.4	0.2	Lavage	+
VP2	7.4	2.4	Plasma	-
VP4	7.4	0.2	Plasma	-
VGS1	2.3	0.2	Gastric Simulans	+
VGS5	2.3	0.5	Gastric Simulans	+

SDS PAGE with Autoradiography. SDS polyacrylamide gel electrophoresis (SDS PAGE) was performed on the products of the reactions of the biological fluids and proteins with TDI. In this case the separation is based on size and is performed on a denatured form of the protein. Coupled with autoradiography this technique supports the observation of covalent association of the 14C-labeled TDI with the protein and it also demonstrates intra- and inter-molecular structural changes as a result of covalent modification by TDI. The following figures (3-6) show groupings of samples on each gel that demonstrate separate effects of TDI on proteins and biological fluids.

Vapor exposure - Effect of position on plate. During the vapor phase exposures samples were placed in tissue culture plates and and exposed to a dynamic vapor passing over the plate in a chamber. Samples from each well were analyzed by SDS PAGE to determine whether uniform reactions took place across the plate. The results show no positional effect on the modification of the RSA.

Vapor Exposure: Effect of pH and Composition. SDS PAGE analysis was performed to determine the effect of pH and composition of different biological fluids on the vapor-phase reaction with TDI. Figure 3A shows the SDS-PAGE electropherogram of the separation of molecules in the various biological fluids based on molecular weight. Figure 3B is the corresponding autoradiogram of the SDS-PAGE gel. The autoradiogram shows that RSA is heavily modified at pH 7.4 (lane 1) and negligibly modified at pH 2.3 (lane 2). Plasma shows modification of a limited number of proteins (lane 5) and lavage fluid shows significant labeling of a protein migrating at a molecular weight corresponding to serum albumin (lane 6). A similar analysis shows that gastric simulans at pH 2.3 results in no modification of the protein fraction (Figure 4). The results of the SDS-PAGE analysis of RSA at pH 2.3 are consistant with the molecular sieve (Centricon) results given in Table V.

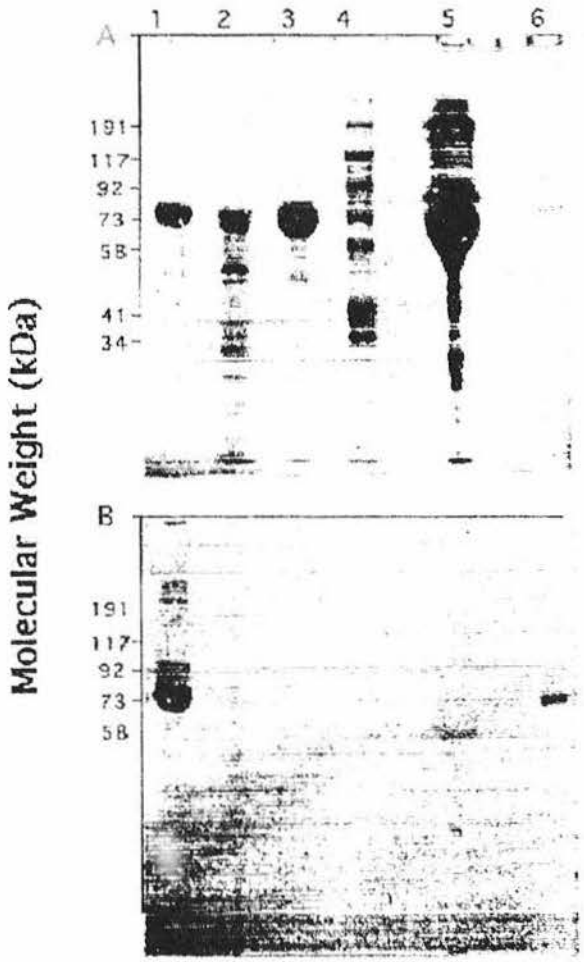

Figure 3. SDS-PAGE analysis of biological materials modified by 0 21 ppm vapor-phase exposure to 14C-TDI. (A) Coomassie Blue stained SDS-PAGE gel; (B) Autoradiogram corresponding to gel in (A). Samples analyzed are; lane 1: RSA modified at pH 7.4; lane 2: RSA modified at pH 2.3, lane 3: unmodified RSA, lane 4: molecular weight standards (alpha-macroglobulin, 190K; beta-galactosidase, 125K; fructose-6-phosphate kinase, 88K; pyruvate kinase, 65K; fumarase, 56K; lactate dehydrogenase, 38K; triosephosphate isomerase, 33K), lane 5: Plasma modified at pH 7.4, and lane 6: lavage fluid modified at pH 7.4.

Vapor Exposure: Effect of Vapor Concentration. SDS PAGE analysis was performed for direct comparison of conjugate products generated at different vapor concentrations of TDI. Figure 4 shows that there is a direct correlation between the concentration of the vapor during exposure and the extent of modification as measured by the intensity of the bands in the autoradiogram (Figure 4B) of the SDS-PAGE gel.

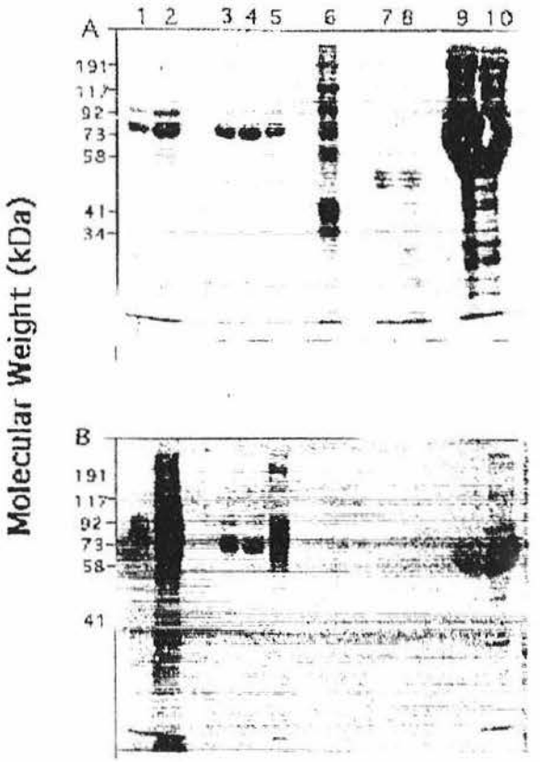

Figure 4. SDS-PAGE analysis of biological materials modified at different vapor concentrations of 14C-TDI. (A) Coomassie Blue stained SDS-PAGE gel; (B) Autoradiogram corresponding to gel in (A). Samples analyzed are; lanes 1 and 2: Lung lavage modified at 0.21 and 1.8 ppm respectively; lanes 3, 4 and 5: RSA modified at 0.55, 0.21 and 1.8 ppm, respectively; lane 6: molecular weight standards (same as Figure 3); lanes 7 and 8: Gastric simulans modified at 0.21 and 0.55 ppm, respectively; lanes 9 and 10: Plasma modified at 0.21 and 1.8 ppm, respectively.

Liquid Exposure: Effect of Concentration. SDS PAGE analysis (Figure 5) of the products of the reaction of RSA at different ratios of TDI to protein under liquid administration conditions. The separation shows that as the ratio of TDI:Protein increases, the apparent molecular weight of the RSA decreases and new bands corresponding to dimeric RSA begin to appear. These observations are consistent with increasing intramolecular crosslinking with increasing TDI concentration as well as increasing intermolecular crosslinking of the RSA molecules. The corresponding autoradiogram (Figure 5B) supports the increased incorporation of the labelled TDI with increasing TDI:Protein ratio.

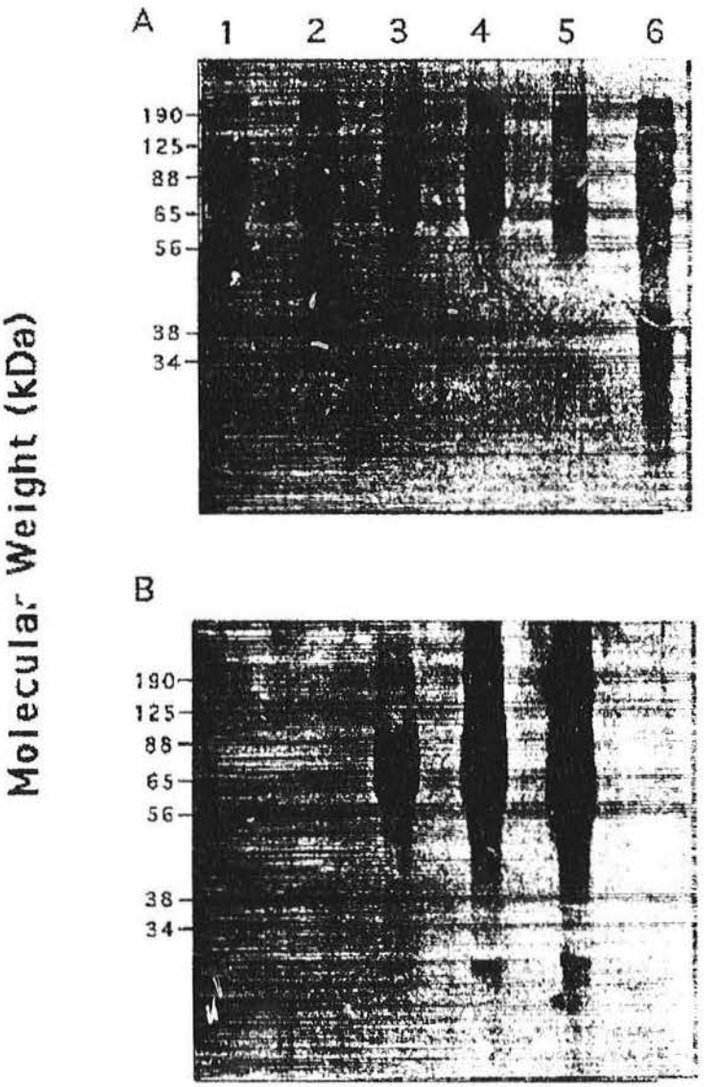

Figure 5. SDS-PAGE analysis of RSA modified at increasing ratios of 14C-TDI:Protein. (A) Coomassie Blue stained SDS-PAGE gel; (B) Autoradiogram corresponding to gel in (A). Samples analyzed are; lane 1: RSA; lane 2: RSA modified at a molar ratio of 0:1 (TDI:Protein); lane 3: RSA modified at a molar ratio of 1:1 (TDI:Protein); lane 4: RSA modified at a molar ratio of 10:1 (TDI:Protein); lane 5: RSA modified at a molar ratio of 50:1 (TDI:Protein); lane 6: Molecular weight standards (Same as Figure 3).

Affinity chromatography of Reaction Products. To determine whether modification of serum albumin had an effect on its structure and function, modified albumins were applied to a Blue Agarose affinity column that specifically binds non-denatured serum albumin. The percentage of modified albumin that does not bind to the column is an index of the alteration of the albumin structure due to TDI modification. Table VIII lists the samples which were subjected to Blue Agarose affinity chromatography and the percentage of albumin which did not bind to the column for each sample tested. Figure 6 gives a representative chromatogram for the elution of RSA that has been modified by vapor exposure to 0.2 ppm TDI for one hour.

TableVIII. Analysis of Blue Agarose Affinity Chromatography

Sample	Name	pH	Mode of Addition	Molar Ratio TDI Added / Protein	% of 14C Associated with >10kDa	% of >10kDa Fraction not Bound to Blue Agarose(peak A, Figure 7)
RSA	control	7.4	liquid	-	-	9.8a
Plasma	VP4	7.4	vapor	0.0019	99	41.8
RSA	VR1	7.4	vapor	2.53	97	96.9
RSA	VR5	7.4	vapor	0.36	99	49.9
RSA	VR9	2.3	vapor	0.50	8.1	12.7
Lavage	VL5	7.4	vapor	0.019	71.6	79.2
GPSA	LG1	7.4	liquid	0.96	91	76.4
GPSA	LG2	7.4	liquid	30.5	91	99.0

a. Measured value based on absorbance at 280 nm of bound and not-bound albumin.

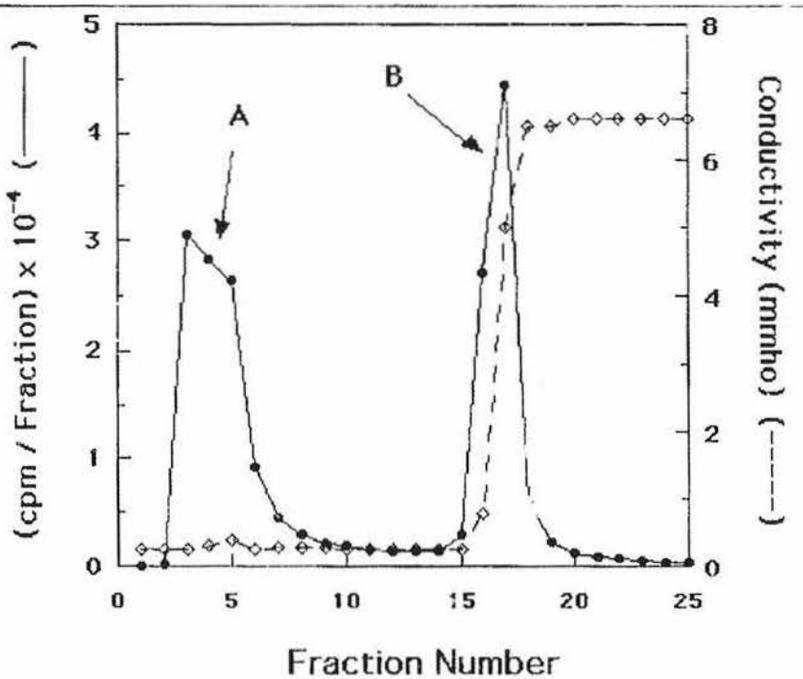

Figure 6. Representative profile from Blue Agarose affinity chromatography of RSA modified by TDI vapor at a concentration of 0.2 ppm for one hour. The first peak (A) represents unbound modified protein while the second peak (B) represents albumin like material that retains its affinity for the albumin affinity column. The sample was first processed through molecular sieve analysis to remove any low molecular weight (<10kDa) labeled materials. The conductivity curve (----) shows where the buffer was changed to release affinity bound albumin.

Summary of Uptake and Incorporation.

Liquid modification. Modification of RSA by TDI correlates linearly with the concentration of TDI added to the reaction mixture. The slope of the correlation (0.906) indicates a very efficient reaction between protein (RSA) and TDI over a wide range of TDI:protein ratios. Thus the reaction of TDI with protein competes successfully with the hydrolysis of the diisocyanate to the diamine over the range of concentrations tested.

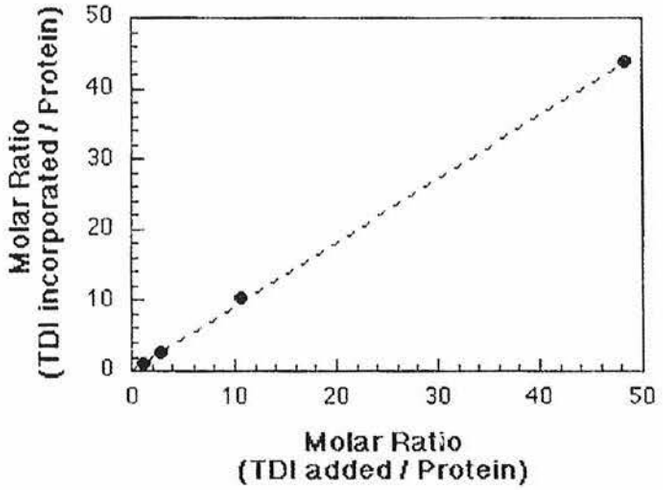

Figure 7. Correlation between the molar ratio of TDI added/Protein and TDI incorporated/Protein. Determination of TDI bound was made after molecular sieve fractionation of the reaction products generated by liquid phase reaction of pure RSA at pH 7.4. The regression analysis of the correlation data yielded the equation y=0.231 + 0.906x (r2=0.999).

Vapor modification. Uptake of the 14C-label into the aqueous phase from the vapor phase during vapor exposure is linear with exposure concentration and independent of biological fluid tested (Figure 8 and Figure 9).

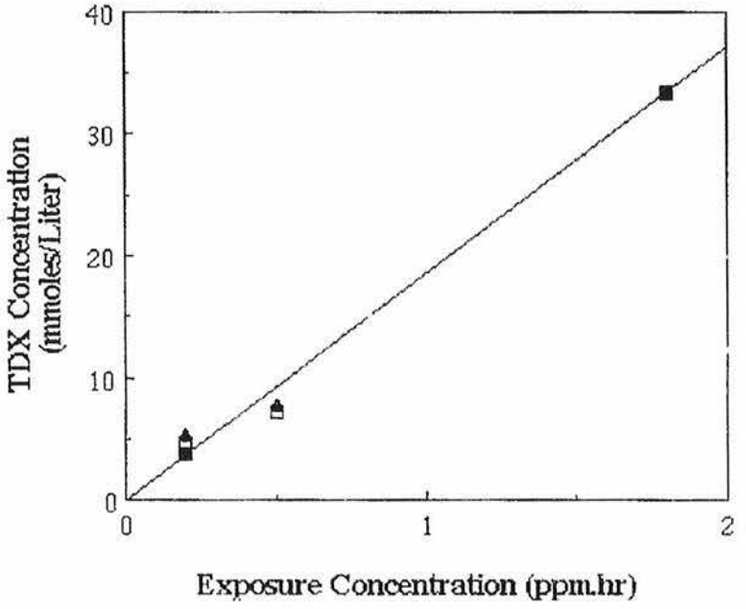

Figure 8. Uptake of 14C-TDX (TDI + TDA) into pH 7.4 aqueous phase as a function of the vapor phase exposure concentration. Pure protein (rat serum albumin, filled triangles) and biological fluids (gastric simulans, open squares; lung lavage, filled squares) were exposed to vapor phase TDI. Concentration of TDX (TDI + TDA + TDI conjugates) in aqueous phase following vapor exposure was determined by liquid scintillation analysis. The line represents a linear regression analysis of the data.

Based on the molecular sieve fractionation data, the moles of TDI incorporated into pure RSA is calculated and found to be linearly related to the exposure concentration (Figure 9). The relationship between the moles of TDI taken up in the

aqueous phase with the moles of TDI incorporated into protein is given in Table IX. The effect of vapor modification on pure albumin is demonstrated by the loss of the more highly modified protein's ability to be recognized by an albumin affinity column (Blue Agarose).

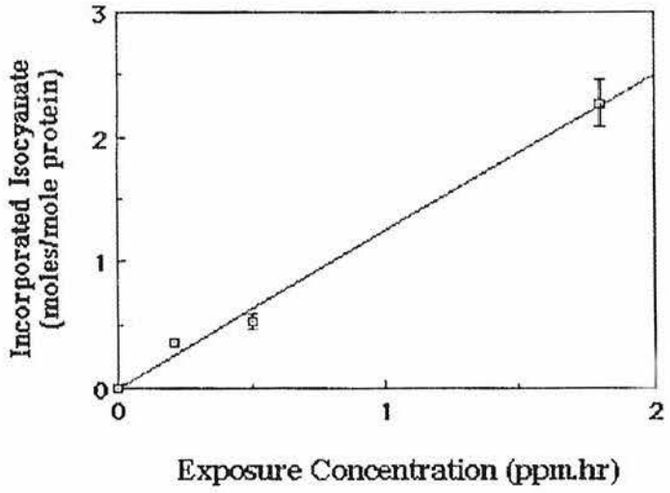

Figure 9. Incorporation of 14C into protein as a function of exposure concentration in the vapor phase.

Table IX. Summary of incorporation of 14C-TDI into pure RSA

Exposure Conc (ppm)	Molar Ratio Total TDI:Protein in aqueous phase (1)	Molar Ratio TDI incorporated:Protein (2)	Fraction of Protein retaining albumin structure (3)
0.21	0.358 ± 0.01	0.358 ± 0.01	0.437
1.79	2.267 ± 0.19	2.199 ± 0.18	0.027

1. Based on 14C-TDI uptake in aqueous phase following one-hour vapor exposure.
2. Based on centricon filtration of protein following vapor exposure in aqueous phase.
3. Based on Blue Agarose affinity binding of native serum albumin

Figure 10 summarizes the combined results showing the separation of high molecular weight conjugates (>10 kDa) (filled section of bars) formed during the reactions and the fraction of low molecular weight species (<10 kDa) (hatched portion of bars) remaining after reaction of each component under the different conditions. In summary, at low pH there was no significant labeling of protein components by TDI, there were differences in the incorporation of label between different biological components at comparable exposure concentrations, and there did not appear to be any differences in incorporation resulting from varying the concentrations of TDI in the reaction.

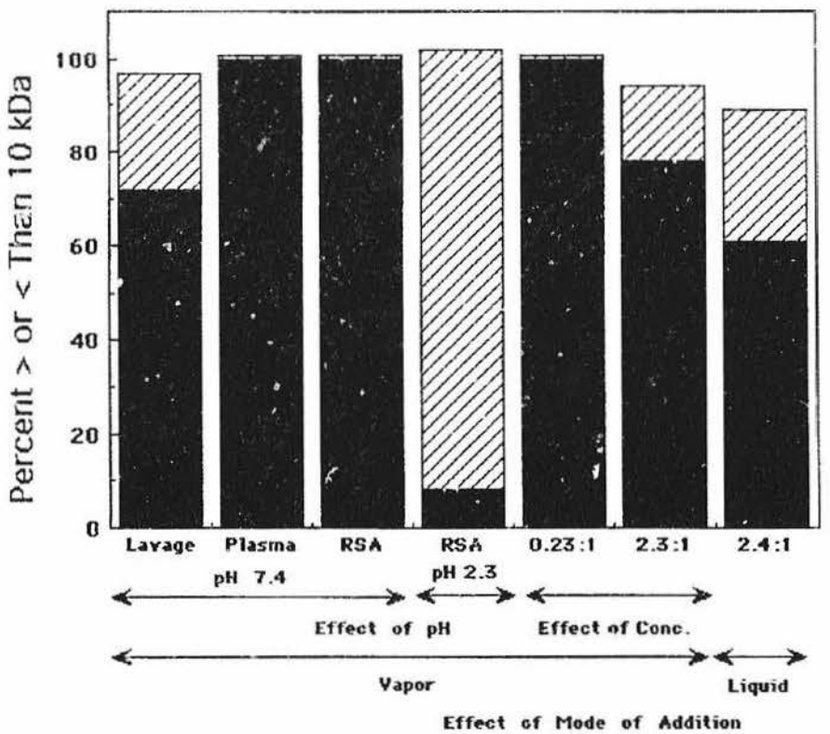

Figure 10. Summary of molecular sieve fractionation data showing effect of reaction composition (lavage, plasma, RSA), pH (7.4 and 2.3), concentration (0.23:1 and 2.3:1 molar ratio (TDI:Protein)) and mode of addition (vapor and liquid). The filled portion of each bar represents the percent of the reaction mixture that was greater than 10 kDa. The hatched portion of each bar is the fraction that is less than 10 kDa.

Summary of Structural Alterations.

Affinity Chromatography. Blue Agarose affinity chromatography was used to assess the loss of albumin binding function as a function of the extent of modification. It was observed that there was a direct correlation between the degree of modification, moles of 14C label incorporated per mole of protein, and the extent to which the albumin component is recognized by the affinity ligand Reactive Blue 4. This would indicate that a high affinity site for the TDI is in the binding site for the Reactive Blue 4 ligand on serum albumin. Thus TDI could be classified as an affinity label for serum albumin which could contribute to the high degree of reactivity of TDI with biological fluids and successful competition with hydrolysis as noted by the low levels of low molecular weight species (< 10 kDa) in the reaction mixture following either vapor or liquid reactions. The concentration data given in Figure 10 would suggest however, that there is a saturation level above which low molecular weight components begin to appear in the reaction mixture.

SDS PAGE Analysis. Several observations can be made from the SDS PAGE analysis of the reaction mixtures. This technique measures the degree to which intra- and inter-molecular reactions take place with each of the component mixtures and each of the reaction modes. These summary observations include:

o Increased modification caused a shift in electrophoretic mobility to lower molecular weight followed by appearance of high molecular weight (dimer, trimer etc) protein species at high concentrations of TDI.

o Reaction at lower pH (pH2.3, gastric simulans) did not alter the structure of molecules nor result in incorporation of TDI into macromolecules.

o There was protein modification of all biological samples at pH 7.4 and none at pH 2.3.

o Vapor modification of proteins showed significant structural effects on proteins to that seen by liquid modification.

DISCUSSION

The effect of the method of administration has often been not addressed in the design and evaluation of toxicological studies with chemically reactive compounds. In addition to the usual metabolic pathway reactions, the test material might also react with the dosing vehicle or any liquid or solid component in the test system (*in vivo*: at tissue surfaces; *in vitro*: with cells, buffer systems etc.). Differences in results or even in the toxicity profile might be the consequence. Isocyanates are one group of highly reactive compounds that have been the focus of numerous *in vitro* and *in vivo* studies. A high degree of reactivity in aqueous systems and with many functional groups of biological macromolecules has been demonstrated (Brown *et al.*, 1987; Gahlman *et al.*, 1993). The question also arises as to whether the method of isocyanate administration alters its reactivity and fate. Differences in bioavailability and metabolism of TDI given to rats orally or via inhalation have been demonstrated (Timchalk, *et al.*, 1994). Equivocal data on mutagenicity and difficulties in interpretation of immunotoxicity results may also be attributable to variable reactions. Differential carcinogenicity data has also been reported for this compound which may be correlated to exposure route (Dieter, 1990; Loeser, 1983). While other methods of *in vivo* dosing are more easily quantifiable, the inhalation route has been documented as the predominant route of workplace exposure (Rampy, *et al.*, 1983). With TDI, or any other reactive compound, it is reasonable to assume that endpoint analysis may be affected by exposure method, and therefore methods that most closely parallel workplace exposure would be optimal.

Based on the high degree of isocyanate reactivity in TDI, it has been hypothesized that upon delivery to aqueous environments such as the airway, GI tract or buffered protein solutions, hydrolysis and/or conjugation reactions may take place with biological macromolecules. The present study was initiated to investigate the effect of administration method *in vitro* at the biochemical level and thereby evaluate possible differences in reactivity and fate. The use of dynamic vapor exposure of *in vitro* test samples was tested as a reaction system which more closely parallel inhalation reaction conditions. Comparison of this vapor exposure system with the widely used organic solvent/water liquid delivery method was also performed.

Vapor exposures of purified protein solutions and biological fluids were performed with 14C-labeled TDI at average vapor concentrations of 0.21, 0.55 and 1.8 ppm (Table II). To parallel these studies, liquid mixing experiments with 14C-TDI acetone solutions were also conducted. The benefit of using the labeled compound is the ability to quantitate uptake into the test samples as well as to characterize the end-products at a high degree of sensitivity. For both methods, the state and concentration of the TDI was assayed to assure the integrity of the starting material.

Following vapor exposure or liquid mixing, the 14C content of each sample was analyzed. For the vapor samples, the results shown in Table IV illustrate that a uniform absorption of the TDI vapor across the air/water interface occurred. The variations in total 14C uptake may be due to solubility differences in lavage and low pH samples as well as a reflection of partition coefficient differences. Positional differences were not observed either across the plates nor within rows, verifying that the vapor exposure of each test system was comparable and any differences are not due to exposure variation.

Biochemical characterization of the vapor-modified samples by several methods was performed to define further the form of the radioactive material in the reaction mixtures. Thin layer chromatography showed that a number of radioactive components were present in the vapor and liquid samples. Molecular sieve fractionation separated the components into high (> 10 kDa) and low (<10 kDa) molecular weight fractions. The rationale for the experiment was that if the TDI was hydrolyzed to the free amine (TDA) or reacted with a low molecular weight peptide or protein, the radioactivity would be recovered in the filtrate (<10kDa) fraction. If, however, reaction with biological macromolecules greater than 10 kDa took place, then the radioactivity would be recovered in the retentate (>10kDa) fraction. Using this method, the effects of sample composition and environment on biochemical reactivity were quantitatively determined (Table V). The data demonstrates that for both plasma and RSA samples at pH 7.4, greater than 99% of the sample radioactivity was associated with the retentate fractions following vapor exposure. However, a decrease in the percentage of radioactivity in the retentate fractions was found for the lavage and to an even greater extent for the RSA samples at pH 2.3.

The Effect of Sample Composition on TDI Reactivity. Considerable differences were observed in reaction products in plasma or protein solutions as compared to the lavage samples (Table V). The fact that a significant fraction (29%) of the reaction mixture was low molecular weight products and that a major component of that fraction was a TDA co-migrating, radioactive component suggests that the potential for the hydrolysis reaction to occur in the airways was present. However, isolated lavage fluid does not accurately represent the reaction environment that is actually present in

the airway. The lavage fluid contained a variety of different cell populations, surfactant components and enzymes active in xenobiotic metabolism (Henderson, *et al.*, 1990). The protein concentration in a buffered lavage solution was lower. The lipid content was also diluted by the buffer and the non-extractable airway cells and their components were absent from the model system. Because the conjugation reaction is bimolecular and was competing with the hydrolysis reaction, the concentration of each component is important. In addition, the choice of reaction path (conjugation vs hydrolysis) would be dependent on the solubility of the reagent and reactant in the solvent state. The presence of surfactant lipids in lavage dramatically alters the environment of the reaction conditions compared to plasma or aqueous pure protein solutions. The effect that each of these factors has on the reaction of TDI in the airway was not determined. Inhalation exposure studies in guinea pigs using 14C-labeled isocyanates have shown by tissue autoradiography that reaction with tissue-associated airway proteins does occur (Kennedy, 1990; Kennedy *et al.*, 1993). There may also be thresholds of concentration where certain target molecules and reaction pathways become saturated and others activated. The effect of concentration on TDI *in vitro* reactivity has been shown in this study where at the higher vapor concentration, the intensity of labeling increases but the selectivity decreases as indicated by the increased number of additional, labeled bands in the SDS-PAGE (Figure 4).

The Effect of pH on TDI Reactivity. A more dramatic decrease in the level of conjugated products was observed as an effect of low pH. At pH 2.3, the percentage of high molecular weight radioactive material in the RSA samples represented only 8% of the total composition. This decrease in conjugation at low pH may be due to the protonation of the potential reaction sites on the functional groups of the protein, making them unavailable for reaction with TDI. Altered rates of hydrolysis may also be contributing to the decrease. In any case, the decreased level of conjugate formation at low pH may have significant impact on the fate of the compound *in vivo*. Timchalk, *et al.*, (1994) have shown that when TDI is delivered by the oral route, the metabolic profile is similar to that of TDA metabolism and that the distribution and fate of the radiolabel is different from results obtained following inhalation exposure. This suggests that the acidic environment of the GI tract favors the hydrolysis to TDA. Biochemical characterization of the blood-associated radioactivity from these exposures showed that in the samples from rats dosed by inhalation, high molecular weight conjugates predominated whereas, low molecular weight products were increased in the samples from rats dosed orally (Kennedy, *et al.*, 1993). Jeffcoat (1985), modeled the reaction of TDI with stomach contents *in vitro* and showed the rapid loss of the isocyanate group, again supporting rapid hydrolysis in this environment.

Effect of Method of Administration on TDI Reactivity. The differences observed in TDI reactivity *in vivo* illustrate the importance of considering exposure-related consequences not only for *in vivo* experiments but for *in vitro* studies as well. As demonstrated with the RSA experiments, even a very similar overall adduct ratio does not reflect the differences in the structure of the adducts or conjugates. The results presented in this comparative study of liquid and vapor modification of protein solutions showed that vapor phase modification slightly favors conjugate formation whereas production of low molecular weight products was increased when the material was added as a liquid solution (Table VI). Therefore, in studies where the TDI is added as a neat liquid or dissolved in a solvent, the material tested and results observed may not be due to the isocyanate itself but rather to other reaction products. For isocyanates and other reactive compounds, it is therefore essential to consider the effects of exposure method on the state of the compound and effects produced.

REFERENCES

Brown, W.E.(1987) The chemistry and biochemistry of isocyanates: An overview. In: Current Topics in Pulmonary Pharmacology and Toxicology (M.A. Hollinger, ed.) Elsevier, New York. pp 200-225.

Brown, W.E. Green, A.H. Cedel, T.E. and Cairns, J. (1987) Biochemistry of protein-isocyanate interactions: a comparison of the effects of aryl vs. alkyl isocyanates. Environ. Health Perspect. 72:5-11.

Brown, W.E. and Kennedy, A.L. (1989) TDI Health Assessment Document, U.S. EPA.

Dieter, M.P., Boorman, G.A., Jameson, C.W., Matthews, H.B., and Huff, J.E. (1990). The Carcinogenic Activity of the Commercial Grade of Toluene Diisocyanate in Rats and Mice in Relation to the Metabolism of the 2,4- and the 2,6-TDI Isomers. *Toxicol. and Industrial Health* **6**: 559-621.

Gahlmann, R. Herbold, B. A. Ruckes, and K. Seel (1993) Untersuchungen zur stabilitat aromatischer diisocyante in dimethylsulfoxid (DMSO): toluylendiisocyanat (TDI) und diphenylmethandiisocyanat (MDI) im Ames-test. Zbl Arbeitsmed. 43:34-38.

Herbold, B. Haas, P. Seel, K. and Walber, U. (1998) Studies on the effect of the solvemage. in Toxicology of the Lung (D.E. Gardner, J.D. Crapo, and E.J. Massaro, eds.) Raven Press; New Yor, pp 239-268.

Jeffcoat, A.R. (1985). Disposition of 2,6-Toluene Diisocyanate in Fischer 344 Rats. *Research Triangle Institute (RTI) Report to the NTP*. Report No. RTI/2227/00-06P.

Karol, M.H. and Alarie, Y.C. (1978) Hapten-specific respiratory hyper-sensitivity in guinea pigs. *Amer. Ind. Hyg. Ass. J.* **39**:546-556.

Kennedy, A.L. Timchalk, C. and Brown, W.E. (1992) The effect of exposure method on the reactivity of toluene diisocyanate. *The Toxicologist* **12**:163.

Kennedy, A.L. (1990) The in vivo reactivity of inhaled isocyanates: comparative analysis of cellular and molecular targets. Ph.D. Thesis, Carnegie Mellon Univ.; Pittsburgh, PA. Chapt 10.

Kennedy, A.L. Singh, G. Alarie, Y. and Brown, W.E. (1993) Autoradiographic analysis of guinea pig airway tissues following inhalation exposure to 14C-labeled methyl isocyanate. *Fundam. Appl. Toxicol* **20**:57-67.

Kennedy, A.L., Stock, M.F., Alarie, Y.A. and Brown, W.E. (1989). Uptake and Distribution of 14C during and following Inhalation Exposure to Radioactive Toluene Diisocyanate. *Toxicol. Appl. Pharmacol.* ***100***: 280-292.

Loeser, E. (1983). Long-term Toxicity and Carcinogenicity Studies with 2,4/2,6-Toluene Diisocyanate (80/20) in Rats and Mice. *Toxicol. Lett.* **15**: 71-81.

Mapp, C.E., Graf, P.D., Boniotti, A., and Nadel, J. (1991). Toluene Diisocyanate Contracts Guinea Pig Bronchial Smooth Muscle by Activating Capsaicin-Sensitive Sensory Nerves. *J. Pharm. Exper. Therap.* **256**: 1082-1085.

Marcali, K. (1957) Microdetermination of toluene diisocyanate in atmosphere. *Anal. Chem.* **29**: 552-558.

NCI (1979). Bioassay of 2,4-Diaminotoluene for Possible Carcinogenicity. *National Cancer Institute, Technical Report Series No. 162*, CAS No. 95-80-7, NCI-CG-TR-162, U.S. Department of Health, Education and Welfare.

NIOSH (1978) Criteria for a recommended standard... occupational exposure to toluene diisocyanate. Rockville, MD: US Department of Health, Education and Welfare; DHEW(NIOSH) publication no. 78-215.

Patterson, R., Hargreave, F.E., Grammer, L.C., Harris, K.E., and Dolovich, J. (1987). Toluene Diisocyanate Respiratory Reactions I. Reassessment of the Problem. *Int. Archs. Allergy Appl. Immun.* **84**: 93-100.

Rosenberg, C. and Savolainen, H. (1985). Detection of urinary amine metabolites in toluene diisocyanate exposed rats. *J. Chromat.* ***323***: 429-433.

Schroeder, R. and Moore K.D. (1985) A reverse phase liquid chromatographic determination of hexamethylene diisocyanate in inhalation chamber atmospheres. Mobay Toxicology Report, No. 639.

Timchalk, C. Smith, F.A. and Bartels, M.J. (1994) Route-dependent comparative metabolism of [14C]toluene 2,4-diisocyanate and [14C]toluene 2,4-diamine in Fischer 344 rats. *Toxicol. Appl. Pharmacol.* **124**:181-190.

B 11

CERTIFICATE OF AUTHENTICITY

THIS IS TO CERTIFY that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming.

Date produced 09 28 99 Mary Turbeck
(Month) (Day) (Year) Camera Operator

Place Syracuse New York
(City) (State)

AMTEK
corp